THE PENETRATION OF RADIOACTIVE PHOSPHATE INTO MARINE EGGS [1, 2]

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Needham and Needham (1930) showed that the gastrulae and plutei of the echinoderm *Dendraster excentricus* have a higher total phosphate content than that of the unfertilized eggs. These authors suggested that the increased phosphate content of the larvae was related to the formation of the skeletal spicules.

Brooks (1943a) obtained results, using radioactive phosphate, which indicated that soon after first cleavage in the fertilized eggs of *Arbacia punctulata,* the intake of radiophosphate was accelerated. During the winter of '46 to '47 these experiments were repeated using more refined methods and the eggs of several different species of sea urchins, as well as the eggs of the gephyrean worm, *Urechis caupo.* The results are presented in this paper. Radiophosphate was found to enter the fertilized eggs of sea urchins more than one hundred times faster than it entered the unfertilized eggs (Brooks and Chambers, 1948). There was no evidence for alternating phases of intake and loss of phosphate ions, such as have been reported to occur during the early period of ion uptake by single *Nitella* cells and by uniform populations of egg cells (Brooks, 1939a, 1939b, 1940, 1943a, 1943b). The previously obtained results are to be ascribed to the considerable variability inherent in the methods which had been used (Brooks, 1951).

Independently Abelson (1947), using the eggs of *Arbacia punctulata,* and Lindberg (1948), using the eggs of *Psammechinus miliaris,* demonstrated the relatively more rapid penetration of radioactive phosphate into the fertilized, as compared to the unfertilized, sea urchin egg.

Methods

Materials. Eggs shed from the ovaries of the freshly collected Pacific coast sea urchins *Strongylocentrotus purpuratus, S. franciscanus* and *Lytechinus pictus,* and eggs obtained from the "egg collectors" (MacGinitie, 1935) of the worm *Urechis caupo* were used. The eggs were strained through cheese cloth and washed four times by centrifugation. In each experiment eggs from only a single animal were used, unless otherwise stated.

Conduct of the experiment. Egg suspensions containing 1 ml. eggs/liter sea water were used. Impaired development occurs if the concentration exceeds 5–6

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ml. eggs/liter sea water. The pH of the sea water surrounding the eggs varied from 8.0 to 8.2 throughout the duration of each experiment, and the temperature of the egg suspensions was maintained at 15 ± 0.1° C., except that suspensions of *Lytechinus pictus* eggs were maintained at 20° to 21° C.

The eggs were kept suspended by using a stirrer rotated at 50 r.p.m. P^{32} of high specific activity was added directly to the egg suspensions. The initial concentration of orthophosphate in the suspension fluid, after addition of P^{32}, varied from 50 to 434 μg P/liter (see Protocols). When thoroughly mixed, the homogeneous egg suspension was divided into two lots, one of which was inseminated by adding one drop (0.05 ml.) of solid sperm, directly removed from the testis, to 1000 ml. of suspension. Examination of the eggs shortly after insemination showed approximately two to three spermatozoa at the periphery of each egg. The remainder of the experiment consisted of removing samples of both unfertilized and fertilized eggs at frequent intervals. At the completion of the experiment, the

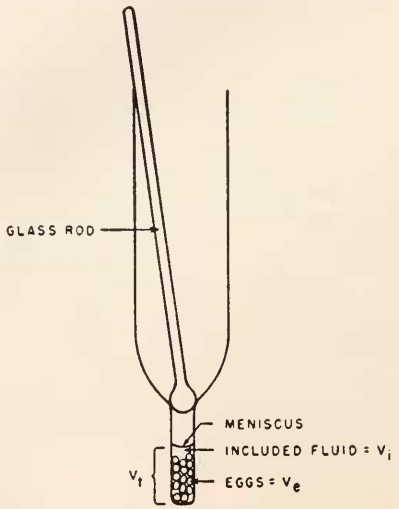

FIGURE. 1. Diagram of Hopkin's tube containing sample of eggs.

eggs previously left unfertilized were inseminated. Development of these eggs, as well as those inseminated earlier, was followed, without diluting the egg suspension, through the pluteus stage for the sea urchin eggs and the trochophore stage for *Urechis* eggs. Development in every case was normal as compared to controls in sea water. This indicated that the P^{32} had been used in concentrations, of the order of 1 to 30 μc/liter, which were below the toxic level.

All experiments were rejected in which (1) the time from insemination to 50 per cent cleavage deviated appreciably from normal, (2) there was undue prolongation of the period between the time when the eggs first started to cleave to the time when cleavage was completed, and (3) less than 95 per cent of the eggs developed to normal swimming embryos.

Removal of egg samples for radioactivity measurements. Each sample of eggs was taken by drawing up one- to two-ml. quantities of the well stirred homogeneous

suspension into a large bore pipette with a wide mouth, and depositing the aliquots in a Hopkin's vaccine tube (Fig. 1) up to the 10.0-ml. mark. The tube was then centrifuged at $86 \times g$ for 60 seconds in a hand centrifuge. This force was just sufficient to drive the eggs into the narrow end of the tube. Within 30 seconds the supernatant was decanted and the fluid remaining within the narrow prolongation of the Hopkin's tube drawn off to a level just above the eggs, using a capillary pipette. The tube was then immediately inverted, and the walls dried with filter paper. The "end point" of penetration of isotope into the eggs was taken as being at the end of the 60 seconds' centrifugation.

The total volume (V_t) of the eggs together with the suspension fluid contained within the narrow prolongation was then determined (Fig. 1). This volume (V_t) amounted to 0.03 to 0.05 ml. in the different experiments. The Hopkin's tube was fixed in a holder fastened to the mechanical stage of a horizontally placed low power microscope provided with an ocular hair line. By operating the stage, the level of the meniscus could be read on the stage scale. Since each tube had been previously accurately calibrated with mercury, the stage scale readings could be converted directly into volume.

After completion of the reading, a thin glass rod with rounded ends was inserted into the tube in order to seal off the mouth of the narrow prolongation (Fig. 1). By holding the rod in place with the index finger, any radioactive solution adhering to the upper walls of the tube was washed out with distilled water without disturbing the eggs at the bottom. After removing the rod, the eggs, together with washings from the bottom of the tube, were transferred to a flat nickel dish 3 cm. in diameter and 3 mm. deep, and dried. The dried material formed a thin even layer on the bottom of the dish, amounting to no more than 1 mg. solids/cm.^{2} of surface. The radioactivity was measured using a Geiger-Müller tube, having a thin mica window 8 cm. in diameter. Samples of the decanted supernatant fluid were dried in the identical dishes and the radioactivity measured.

The question arose as to how accurately the 10.0-ml. aliquots represent the suspension as a whole. This was determined by taking a batch of unfertilized eggs and removing the jelly by several washings. A dozen 10.0-ml. samples were taken as above described in the Hopkin's tubes and centrifuged for ten minutes at $2000 \times g$. The top of the eggs packed in the narrow prolongation of the Hopkin's tubes formed a perfectly straight line, and its level was measured as previously described. The volumes thus obtained were within a maximum range of 0.2 per cent of each other, indicating the validity of the sampling procedure used.

The advantage of the above described method for determining the quantity of radioactive isotope in the eggs is that, by eliminating the necessity for washing the cells, errors which might arise from injury to the eggs and from outward leaching of ions or compounds are avoided.

Egg volume measurements. The mean diameter of fertilized eggs in the early one-cell stage was determined by averaging 25 individual measurements made with a filar micrometer. The unfertilized eggs of the sea urchin are never spherical when freshly removed from the ovaries, and *Urechis* eggs in the unfertilized state are indented on one side. Soon after fertilization the eggs of both species become spherical with only slight changes in volume (Tyler, 1932). The average diameter of *S. purpuratus* eggs is 81.3 μ, *S. franciscanus* eggs 119 μ, and *Urechis caupo* eggs

110 μ. The number of eggs per ml. of suspension was determined as follows. Using a wide-mouthed pipette a sample, approximately 0.2 ml. in volume, was withdrawn from the homogeneous suspension, deposited on a ruled slide, covered with a coverslip, and weighed in order that the volume of the sample could be accurately determined. The total number of eggs on the slide was then counted. This procedure was repeated twice and the results averaged. Knowing the number of eggs in a given mass of sea water and the average diameter of one egg, the total volume of egg protoplasm (Ve) in a 10.0-ml. volume of suspension could be readily calculated.

Egg volume determinations were also carried out by centrifuging the jelly-free unfertilized eggs for 10 minutes at $2000 \times g$. Results obtained by this method were not significantly different from volume determinations carried out by counting the number of eggs in aliquots and measuring diameters.

Calculations. The concentration of P^{32} within the eggs was calculated as follows. Knowing the total volume of eggs with included fluid (V_t) contained within the narrow prolongation of the Hopkin's tube (Fig. 1), and the volume of eggs in 10.0 ml. of suspension (Ve), the volume of the included fluid alone is: $(V_i) = (V_t) - (Ve)$. The included fluid volume (V_i) represents the quantity of fluid exterior to the protoplasmic surface of the eggs. This volume, therefore, includes the space occupied by the egg jelly and the space enclosed within the fertilization membrane, structures which allow free diffusion of phosphate ions. The radioactivity of the included fluid is obtained by multiplying $(V_i) \times$ C.P.M. (counts per minute) of one ml. suspension fluid. Since the radioactivity of the total sample is known, the radioactivity of one ml. of eggs is:

$$\text{C.P.M./ml. eggs} = \frac{(\text{C.P.M. of sample}) - (\text{C.P.M. of included fluid})}{V_e}. \quad (1)$$

Under the conditions of the counting method used, 1 C.P.M. $= 3.6 \times 10^{-6}$ μc P^{32}. Using this conversion factor, the results have been expressed in terms of μc P^{32}/ml. eggs.

Accuracy of method. The following sources of error were taken into consideration in calculating the standard deviation for each determination of the μc P^{32}/ml. eggs (see Tables I and II): the sampling error, error in determining the egg volume (V_e) and the included fluid volume (V_i), error in determining the C.P.M. of the sample, of the background, and of the supernatant fluid. When the radioactivity contributed by the included fluid in each sample is more than half that of the entire sample, the error of the method is considerable. Accuate results are obtained when the concentration of isotope in the eggs exceeds the concentration of isotope in the suspension fluid.

Protocols

Experiment 1. P^{32} was added to a suspension containing 1.00 ml. *S. purpuratus* eggs/liter 120 minutes after the eggs had been removed from the ovaries, and the eggs were inseminated five minutes later. The initial concentration of P^{32} in the sea water was 1.22 μc/liter, and the orthophosphate concentration approximately 71 μg P/liter. First cleavage started 97 minutes after insemination, was 50 per cent complete at 104 minutes and finished at 110 minutes.

Experiment 2. P^{32} was added to a suspension containing 1.09 ml. *S. purpuratus* eggs/liter 135 minutes after removal of the eggs from the ovaries, the eggs being inseminated 12 minutes later. The initial concentration of P^{32} in the sea water

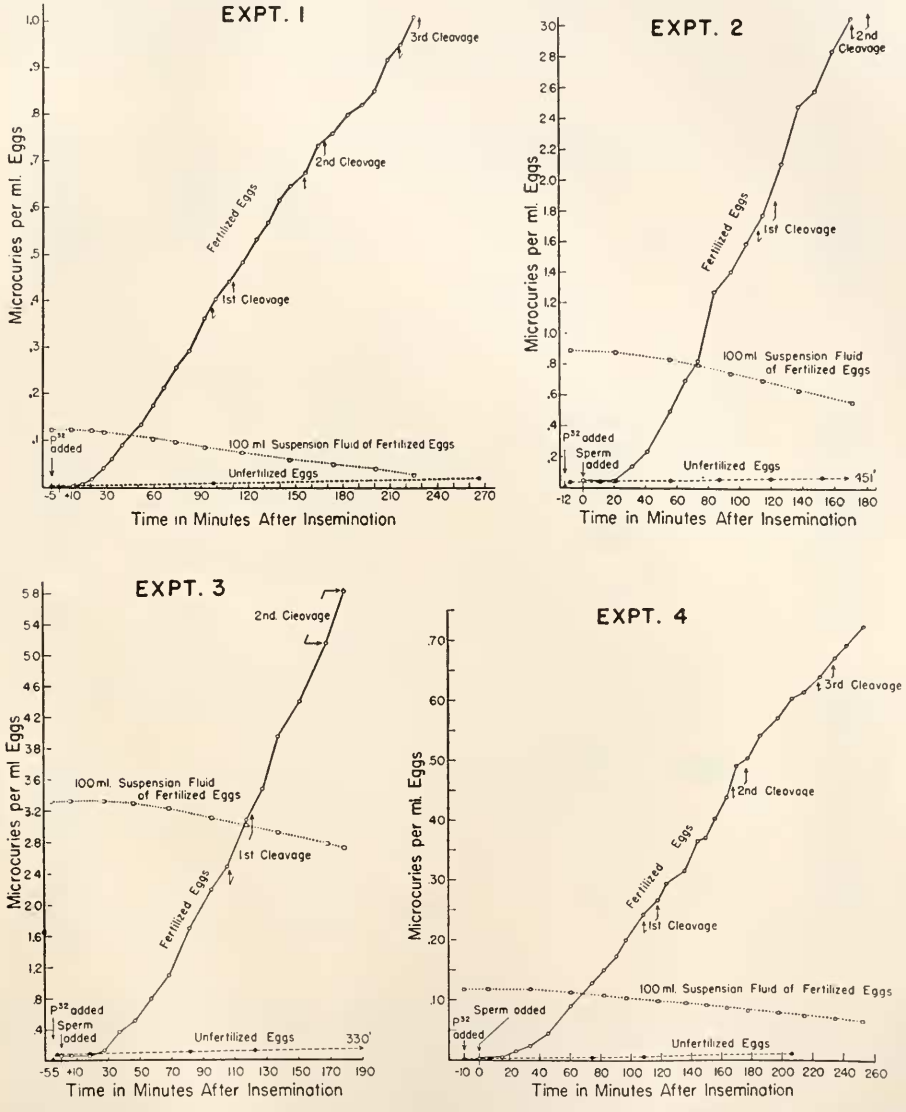

FIGURE 2. Uptake of P^{32} by unfertilized and fertilized sea urchin eggs. Expts. 1, 2 and 3: *S. purpuratus eggs.* Expt. 4: *S. franciscanus eggs.* At beginning of each experiment P^{32} added to suspension and then divided into two lots. Sperm added to one lot at 0 minutes in all experiments—fertilized eggs. The second lot was not inseminated—unfertilized eggs. Ordinates: μc P^{32}/ml. eggs. Abscissae: Time in minutes before and after insemination, 0 = time of insemination. (○——○) fertilized eggs, (●----●) unfertilized eggs, (□····□) μc P^{32}/100 ml. suspension fluid of fertilized eggs.

TABLE I

Uptake of P^{32} by the unfertilized and fertilized eggs of S. purpuratus. Expts. 1, 2, and 3

Time after insemination in minutes	Unfertilized, μc P^{32}/ml. eggs	Fertilized, μc P^{32}/ml. eggs	Suspension fluid of fertilized eggs, μc P^{32}/ml.
	Expt. 1, *S. purpuratus*		
−5.0	P^{32} added to suspension		
0.0	One-half of suspension inseminated		0.00122
8.3	0.00184 ± .0009	0.00111 ± .0009	0.00122
59.6	—	0.175 ± .003	0.00103
92.4	0.00810 ± .0009	0.359 ± .004	0.00085
173.8	—	0.756 ± .006	0.00048
225.1	—	1.01 ± .01	0.00024
266.5	0.0188 ± .0010	—	—
	Expt. 2, *S. purpuratus*		
−12.0	P^{32} added to suspension		
−8.2	0.034 ± .002		
0.0	One-half of suspension inseminated		0.00885
21.0	0.046 ± .002	0.047 ± .003	0.00872
56.0	0.050 ± .002	0.494 ± .009	0.00826
84.4	—	1.27 ± .09	0.00770
87.1	0.063 ± .002	—	—
127.2	—	2.09 ± .14	0.00665
152.0	0.069 ± .003	—	—
171.1	—	3.02 ± .16	0.00560
	Expt. 3, *S. purpuratus*		
−5.5	P^{32} added to suspension		
−2.5	0.0809 ± .0064		
0.0	One-half of suspension inseminated		0.0335
18.0	0.0924 ± .0061	0.0444 ± .0044	0.0333
46.6	—	0.496 ± .071	0.0328
81.5	0.102 ± .007	1.68 ± .09	0.0316
122.5	0.119 ± .006	—	—
128.0	—	3.46 ± .10	0.0294
179.2	—	5.79 ± .12	0.0270

was 8.9 μc/liter, and the orthophosphate concentration approximately 133 μg P/liter. First cleavage started at 113 minutes after insemination, 50 per cent had cleaved at 118 minutes and completed at 123 minutes.

Experiment 3. P^{32} was added to a suspension containing 1.10 ml. *S. purpuratus* eggs/liter 127 minutes after the eggs had been removed from the ovaries, and the eggs were inseminated 5.5 minutes later. The initial concentration of P^{32} in the sea water was 33.5 μc/liter and the orthophosphate concentration approximately 434 μg P/liter. First cleavage started 107 minutes after insemination, was 50 per cent complete at 114.5 minutes and was finished at 121 minutes.

Experiment 4. P^{32} was added to a suspension containing 0.72 ml. *S. franciscanus*

eggs/liter 120 minutes after the eggs had been removed from the ovaries, and the eggs inseminated 10 minutes later. The initial concentration of P^{32} in the sea water was 1.2 μc/liter, and the orthophosphate concentration approximately 59 μg P/liter. First cleavage started 108 minutes after insemination, was 50 per cent complete at 113 minutes and reached completion at 117 minutes.

Experiment 5. P^{32} was added to a suspension containing 1.29 ml. *U. caupo* eggs/liter 180 minutes after removal of the eggs from the animal, and the eggs were

TABLE II

Uptake of P^{32} by the unfertilized and fertilized eggs of S. franciscanus and Urechis caupo. Expts. 4 and 5

Time after insemination in minutes	Unfertilized, μc P^{32}/ml. eggs	Fertilized, μc P^{32}/ml. eggs	Suspension fluid of fertilized eggs, μc P^{32}/ml.
	Expt. 4, *S. franciscanus*		
−10.0	P^{32} added to suspension		
0.0	One-half of suspension inseminated		0.00118
6.3	0.0031 ± .0013	0.0012 ± .0012	0.00118
74.8	0.0038 ± .0014	0.127 ± .005	0.00109
108.2	0.0075 ± .0015	0.240 ± .005	0.00100
163.2	—	0.435 ± .007	0.00086
206.7	0.0094 ± .0015	0.600 ± .009	0.00076
252.0	—	0.718 ± .011	0.00066
	Expt. 5, *Urechis caupo*		
−10.0	P^{32} added to suspension		
−5.0	0.0007 ± .0004		
0.0	One-half of suspension inseminated		0.00089
33.3	—	0.0028 ± .0005	0.00089
43.0	0.0039 ± .0006	—	—
91.5	—	0.0066 ± .0005	0.00088
100.8	0.0056 ± .0006	—	—
176.9	—	0.0145 ± .0006	0.00087
184.1	0.0103 ± .0007	—	—
223.2	—	0.0220 ± .0007	0.00085
266.0	—	0.0369 ± .0010	0.00083
275.0	0.0174 ± .0007	—	—
517.0	—	0.285 ± .005	0.00052

inseminated 10 minutes later. The initial concentration of P^{32} in the sea water was 0.89 μc/liter, and the orthophosphate concentration about 50 μg P/liter. First cleavage was 50 per cent complete at 119 minutes after insemination, second cleavage at 160 minutes and third cleavage at 209 minutes.

RESULTS

Uptake of P^{32} by unfertilized and fertilized eggs

The results of five experiments are presented in Figures 2 and 3. The data are abbreviated in Tables I and II, in which only a few of the determinations are

presented for each experiment. The first column in each table indicates the time when samples of unfertilized and fertilized eggs were removed for radioactivity measurements. P^{32} was added to the suspension of the unfertilized eggs at the beginning of the experiment. Shortly thereafter the first sample was removed, the suspension divided into two lots, and one lot inseminated. In every case the time of insemination is set as zero time. In the second column the quantity of P^{32} which has penetrated the unfertilized eggs after various time intervals is shown. The third column presents the same data for the fertilized eggs. In the fourth column the decrease in concentration of P^{32} in the suspension medium is shown.

Eggs of Strongylocentrotus purpuratus and S. franciscanus. The uptake of P^{32} by the unfertilized eggs is shown in Figure 2, interrupted line with solid circles.

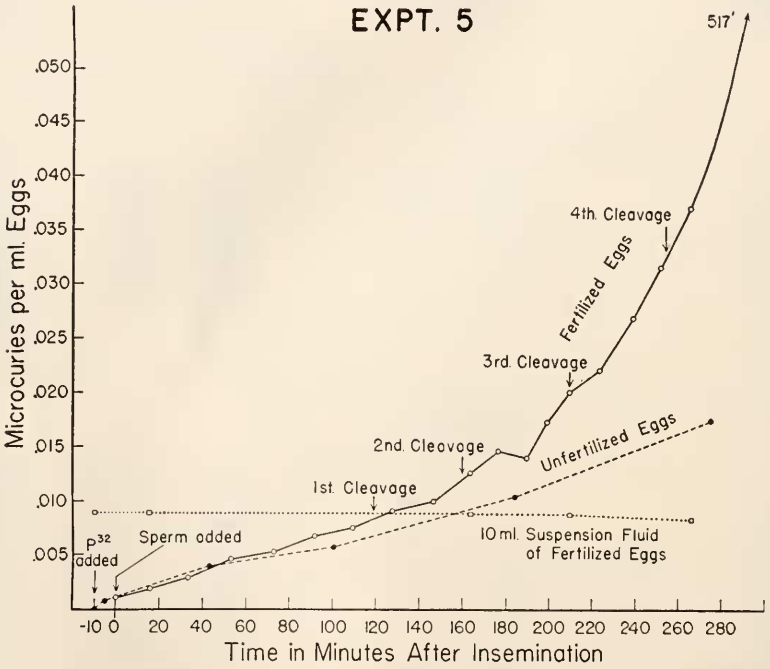

FIGURE 3. Uptake of P^{32} by the unfertilized and fertilized eggs of *Urechis caupo*, Expt. 5. Legend as for Figure 2.

In Experiments 2 and 3 the initial uptake of P^{32}, during the first three to four minutes after adding the isotope, appears to be greater than the uptake in the subsequent 200 minutes. This initial increase, however, undoubtedly does not represent penetration of P^{32} into the eggs, but is an artifact arising from a small error in determining the absolute volume of the eggs, or from the initial absorption of a small quantity of the isotope to the extraneous coats or the surface of the eggs. In Experiments 1 and 4, in which the concentrations of P (as orthophosphate) and P^{32} in the suspension fluid were much less than in Experiments 2 and 3 (see Protocols), the absence of an initial phase of rapid P^{32} uptake is evident. In all

experiments, with the exception of the initial phase in Experiments 2 and 3, the uptake of P^{32} by the unfertilized eggs occurred at a constant rate.

The uptake of P^{32} by fertilized eggs (Fig. 2, solid line with open circles) during the first 10 to 15 minutes following fertilization is identical to the uptake shown by unfertilized eggs. By 15 to 20 minutes following fertilization the rate of uptake increases until by 50 to 60 minutes in *S. purpuratus*, and 80 to 90 minutes in *S. franciscanus* the uptake has reached a maximum rate. Thereafter, except for minor variations, the uptake occurs at a constant rate through the third cleavage. The minor variations in the rate of uptake which occurred were within the range of experimental error. No change in rate of uptake during the cleavage cycles was evident.

Results obtained using the eggs of *Lytechinus pictus* are essentially identical to those obtained using the eggs of the two species of *Strongylocentrotus* (Chambers and Whiteley in Whiteley, 1949).

Eggs of Urechis caupo. The uptake of P^{32} by the unfertilized eggs (Fig. 3, interrupted line with solid circles) was observed to occur at a slow constant rate. The uptake of P^{32} by the fertilized eggs (Fig. 3, solid line with open circles) was essentially identical to that of the unfertilized eggs throughout the period of maturation and the first two cleavages (the eggs, laid in the germinal vesicle stage, do not mature until after insemination occurs). Shortly after the second cleavage the rate at which the fertilized eggs removed P^{32} from the medium increased. Even after the fourth cleavage the rate continued to increase.

Loss of P^{32} from the eggs

Experiments were carried out to determine whether or not P^{32} contained within the eggs is lost to the medium when the P^{32} in the sea water surrounding the eggs is removed. The experiments were carried out using the eggs of *Lytechinus pictus* as follows: Unfertilized and fertilized eggs were exposed to sea water containing P^{32} and approximately 60 μg P as orthophosphate/liter for one hour. Samples of the suspension were then taken to determine the quantity of P^{32} which had entered the eggs, and immediately thereafter the remainder of the suspension was gently centrifuged, the supernatant decanted, and replaced by fresh non-radioactive sea water containing about 60 μg P as orthophosphate/liter. After washing three times by centrifugation, a suspension of the washed eggs was prepared containing 3.0 ml. eggs/liter sea water. Ten-ml. samples of this suspension were removed at various intervals of time for radioactivity determinations. The washing of the eggs was repeated at frequent intervals, in order to remove any P^{32} which may have entered the medium from the eggs. The results are shown in Figure 4. The quantity of P^{32} remaining within the eggs is expressed in terms of the per cent of the quantity of P^{32} within the eggs immediately preceding the first washing. The times when the eggs were washed are indicated by the small arrows in the figures. During careful washing of the eggs, in spite of the greatest precautions it is impossible to avoid destroying or losing some of the eggs. This is particularly true of the fragile unfertilized eggs. Accordingly, the volume of unfertilized eggs in each 10-ml. sample taken was determined by the centrifugal method, and correction made for any loss of eggs which may have occurred during the repeated washings.

The results obtained on the unfertilized eggs (Fig. 4, curves in upper half) reveal that in two experiments 15 per cent. and in one experiment 4 per cent of the P^{32} initially contained within the eggs was lost to the medium during the first 100 minutes after washing was started. The P^{32} continues to be lost at a slow rate over a long period of time. However, the results obtained after 300 minutes from the time the eggs were first washed are open to question, because fertilization and development of these eggs were impaired.

The fertilized eggs are far more resistant to the washing procedure, since they are protected by their fertilization membranes. It was not possible, however, to correct for such losses of eggs as may have occurred, during washing, as egg volumes

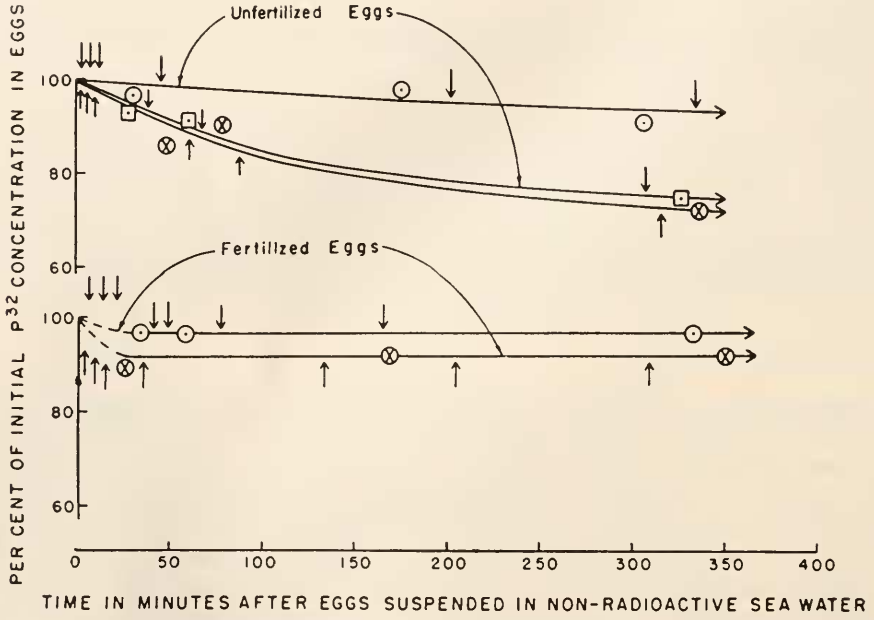

FIGURE 4. Effect of washing unfertilized and fertilized *Lytechinus pictus* eggs containing P^{32} in non-radioactive sea water.

in the samples cannot be determined when the eggs are fertilized. The results of two experiments on fertilized eggs are shown in Figure 4 (curves in lower half). A loss of P^{32} from the samples taken after the first series of washings occurred, but subsequently, no appreciable loss of P^{32} from the eggs was observed. The initial loss of P^{32} is undoubtedly due to the loss of some eggs, for which no correction could be made.

In six experiments carried out using unfertilized and fertilized *S. purpuratus* eggs, similar results were obtained.

The influence of extraneous coats on the uptake of P^{32}

The uptake of P^{32} by the fertilized eggs of *Lytechinus pictus* from which the extraneous coats had been removed was compared to that of normal eggs possessing

all their coats intact. The experiment was performed as follows: A suspension of unfertilized eggs was divided in two beakers. The eggs in one beaker were inseminated. One-half of this suspension was centrifuged, the supernatant discarded and two minutes after insemination, at the time when observation revealed that the fertilization membranes were rising, the eggs were suspended in a mixture of 95 parts 1 *M* urea, pH 8.0, and five parts sea water. The eggs were allowed to settle, and five minutes after insemination the urea solution was decanted and replaced by sea water. The decantations were repeated several times until the eggs had been washed free of the urea solution. Observation of the eggs revealed that the jelly coats and the fertilization membranes had been completely removed and that the hyaline plasma layer did not form (Moore, 1930). When the urea-treated eggs cleaved, they separated into two spherical blastomeres, fastened together only by delicate stalks. The fertilized untreated eggs and the urea-treated eggs cleaved 100 per cent and at the same time. At 50 minutes after insemination, P^{32} was added to all three suspensions, the control unfertilized eggs, the control fertilized eggs, and the urea-treated fertilized eggs. Samples were removed at intervals for radioactivity determinations. The results are shown in Table III. They reveal that in the 25-minute interval during which the uptake of P^{32} was measured, 75 times as much P^{32} entered the fertilized eggs as the unfertilized eggs,

TABLE III

Uptake of P^{32} by unfertilized, normal fertilized and urea-treated fertilized Lytechinus pictus eggs

Condition of eggs	Uptake of P^{32}, in C.P.M./ml. eggs
Unfertilized	2,000
Fertilized, controls	150,000
Fertilized, urea treated	145,000

and that as much P^{32} entered the denuded urea-treated eggs as the control fertilized eggs. Since the urea-treated eggs in sea water may still have possessed a thin but invisible coating of proteinaceous material, at the end of the 25-minute interval these eggs were washed for 10 minutes in three changes of an isotonic mixture of 10 parts 0.53 *M* KCl and 90 parts 0.52 *M* NaCl at pH 6.0 and then suspended in this mixture. Such a treatment should have dissolved away any remaining extraneous material surrounding the eggs (Chambers, 1940). The washing of the urea-treated eggs in the Na/K mixture did not remove an appreciable quantity of P^{32} from the eggs, revealing that no significant quantity of P^{32} is absorbed to the coats, which surround the eggs external to the protoplasmic surface.

Rate of penetration into the eggs

The quantity of P^{32} in μc entering one ml. eggs in a given interval of time, $t_2 - t_1$, may be obtained directly from the graphs. Rates of penetration were determined only for the constant phases of uptake, *i.e.*, from five minutes (t_1) to 200 minutes (t_2) after addition of P^{32} for the unfertilized eggs, from 70 minutes (t_1) to 170 minutes (t_2) after insemination for the fertilized sea urchin eggs, and from 250 minutes (t_1) to 275 minutes (t_2) after insemination for the fertilized *Urechis* eggs.

In spite of a very considerable decrease in the concentration of P^{32} in the medium

surrounding the fertilized sea urchin eggs, the uptake of P^{32} by the eggs remained constant throughout the duration of Experiments 1–4. In experiments on the unfertilized eggs, and the fertilized eggs of *Urechis caupo,* no appreciable change in concentration of P^{32} in the suspension fluid occurred, since only a small quantity of P^{32} penetrated the eggs.

Fertilized eggs of S. purpuratus and S. franciscanus. The decrease in concentration of P^{32} in the medium surrounding the fertilized eggs could be due either to an exchange of P^{32} from the external medium for P inside the eggs, or to an accumulation of P within the eggs, depleting the P in the suspension fluid. Experiments described in this paper reveal that when fertilized eggs, which had been exposed to sea water containing P^{32}, are immersed in radioactive-free sea water containing about 60 μg P/liter no appreciable quantity of P^{32} leaves the eggs. Chambers and White (1949, 1954) have shown that fertilized eggs remove orthophosphate from a medium containing between 10 to 100 μg P/liter at the same rate as the P^{32}.

The specific activity (μc P^{32}/μg P) of the P (as orthophosphate) in the medium, therefore, remains constant throughout the duration of the experiment. Accordingly, the rate of penetration of orthophosphate into the eggs can be accurately calculated as follows:

$$\mu\text{g P/ml. eggs/min.} = \left(\frac{\mu\text{g } P_s}{\mu\text{c } P_s^{32}}\right)\left(\frac{\mu\text{c } P_e^{32} \text{ at } t_2 - \mu\text{c } P_e^{32} \text{ at } t_1}{(t_2 - t_1)}\right), \qquad (2)$$

where μg P_s = initial concentration of orthophosphate in the suspension fluid in μg P/ml., μc P_s^{32} = initial concentration of P^{32} in μc/ml. in the suspension fluid, μc P_e^{32} at t_2 and t_1 = concentration of P^{32} in μc/ml. eggs at the time in minutes t_2 and t_1.

The rates of penetration of orthophosphate, calculated according to equation (2) are shown in Table IV, including three experiments from Chambers and White (1954.)

For the fertilized eggs of *S. purpuratus,* the rate of P uptake, from 70 to 170 minutes after insemination, at 15° C., in four experiments, varied from 0.54 μg P/ml. fertilized eggs/minute at an external orthophosphate concentration of 416 μg P/liter to 0.28 μg P/ml. fertilized eggs/minute at an external orthophosphate concentration of 20 μg (P/liter (Table IV, columns 3 and 5). With a twenty-fold change in concentration of P, only a 1.9-fold change in the rate of penetration of P occurred. As long as the orthophosphate concentration exceeds about 20 μg P/liter, the more dilute the orthophosphate concentration, the greater is the fraction of P in the medium which is absorbed by the fertilized eggs in a given period of time.

The rate of penetration of orthophosphate into fertilized *Strongylocentrotus franciscanus* eggs from 70 to 170 minutes after insemination, at 15° C., in three experiments, ranged from 0.11 to 0.17 μg P/ml. fertilized eggs/minute, with the concentration of orthophosphate in the external medium varying from 63 to 20 μg P/liter (Table IV, columns 3 and 5). The rate of uptake by the fertilized *S. franciscanus* eggs is about half that of *S. purpuratus* eggs.

Unfertilized eggs of S. purpuratus and S. franciscanus. In the experiments carried out with unfertilized eggs, no change could be detected in the concentration

of P^{32} in the suspension fluid. Although the concentration of orthophosphate in the sea water surrounding unfertilized sea urchin eggs remains constant or slowly increases (Chambers and White, 1954), the amount of increase is not sufficient to appreciably alter the specific activity of the orthophosphate in the medium as long as the egg suspension is dilute (1.0 ml. eggs/liter suspension) and the concentration of orthophosphate in the medium exceeds 50 μg P/liter.

Unlike fertilized eggs, unfertilized eggs containing P^{32}, when immersed in non-radioactive sea water, slowly lose P^{32} to the surrounding medium. The rate, however, at which P^{32} is lost from eggs which had been exposed to P^{32} sea water is over a hundred times slower than the rate at which the P^{32} originally entered the eggs. In view of these considerations and the linear P^{32} uptake curves, equation

TABLE IV

Rate of penetration of phosphate into unfertilized and fertilized S. purpuratus, S. franciscanus and Urechis caupo eggs at 15° C.

Experiment	Condition of eggs	Initial and final P conc. μg P/liter susp. fluid	μg P/ml. eggs/min.		Fertilized, μg P/ml. eggs/min. / Unfertilized, μg P/ml. eggs/min.
			Unfertilized	Fertilized	
S. purpuratus,	Unfert.	71	0.0035		
Expt. 1	Fert.	56 to 29		0.30	86
S. purpuratus,	Unfert.	133	0.0035		
Expt. 2	Fert.	122 to 84		0.37	106
S. purpuratus,	Unfert.	434	0.0041		
Expt. 3	Fert.	416 to 359		0.54	132
*S. purpuratus**	Unfert.	78	0.0026		
	Fert.	78 to 20		0.28	106
S. franciscanus,	Unfert.	59	0.0015		
Expt. 4	Fert.	55 to 42		0.17	113
S. franciscanus†	Fert.	53 to 20		0.11	—
S. franciscanus‡	Fert.	63 to 20		0.14 ot 0.17	—
U. caupo,	Unfert.	50	0.0033		
Expt. 5	Fert.§	47 to 46		0.024	7

* From Chambers and White (1954), Expts. 5 and 6.
† From Chambers and White (1954), Expt. 7.
‡ From Chambers and White (1954), Expt. 8.
§ After third cleavage.

(2) may be used to calculate the rate of entry of orthophosphate into unfertilized sea urchin eggs.

In the case of the unfertilized eggs of *S. purpuratus* the rate of penetration of orthophosphate at 15° C. in three experiments varied between 0.0041 to 0.0026 μg P/ml. unfertilized eggs/minute with concentration of P (as orthophosphate) in the medium ranging from 434 to 71 μg P/liter (Table IV, columns 3 and 4). For the unfertilized eggs of *S. franciscanus* the rate of P uptake, in one experiment, was 0.0015 μg P/ml. unfertilized eggs/minute, at an external orthophosphate concentration of 59 μg P/liter.

Fertilized and unfertilized Strongylocentrotus eggs compared. As shown in Table IV, column 6, phosphate penetrates fertilized *S. purpuratus* eggs 86 to 132 times more rapidly than unfertilized eggs.

In *S. franciscanus* eggs, approximately a 113-fold increase in rate occurs after fertilization.

Eggs of Urechis caupo. The rates of penetration of orthophosphate into the unfertilized and fertilized eggs were arbitrarily calculated according to equation (2). In view of the slow rate of P^{32} uptake, the inappreciable change in concentration of P^{32} in the suspension fluid, and the dilute egg suspension, it is probable that the use of equation (2) is justified.

Phosphate penetrates the unfertilized eggs of *Urechis caupo* and *S. purpuratus* at about the same rate (Table IV, columns 3 and 4). The important difference between the *Urechis* and the sea urchin egg is that in the former species, after fertilization, no increase in the rate of P uptake occurs. After the *Urechis* eggs have undergone second cleavage, however, the rate starts to increase, attaining a rate 7 times that of the unfertilized eggs by the time of fourth cleavage (Table IV, column 6). Even by this time the rate has not reached its maximal level (Fig. 3).

Discussion

When fertilized sea urchin eggs which had been exposed to sea water containing P^{32} are immersed in radioactive free sea water, no appreciable quantity of P^{32} leaves the eggs. Furthermore, fertilized sea urchin eggs remove orthophosphate from sea water at the same rate as P^{32} (Chambers and White, 1949, 1954). These data reveal that the entry of P^{32} into the fertilized sea urchin eggs measures the rate at which phosphate accumulates within the cells.

On the other hand, when P^{32} is added to a suspension of unfertilized eggs, no appreciable change in concentration of P^{32} occurs in the suspension fluid. The concentration of orthophosphate in the suspension fluid surrounding unfertilized eggs remains constant or slowly increases (Chambers and White, 1954). When unfertilized eggs, containing P^{32}, are immersed in non-radioactive sea water, P^{32} slowly washes out into the external medium. On the basis of these data, the conclusion may be made that the uptake of P^{32} by unfertilized eggs measures the rate at which phosphate enters the eggs, presumably by exchange, at the same time that the internal concentration remains constant, or even decreases. The change from the unfertilized to the fertilized state, therefore, involves not only a change in magnitude but also a reversal of "driving forces." Although the two-fold increase following fertilization in permeability to water (Lillie, 1916) and non-electrolytes (Stewart and Jacobs, 1932) may contribute to the striking increase in uptake of orthophosphate which follows fertilization, it is probable that changes in "driving forces" play the dominant role.

Of interest is the relatively constant rate at which phosphate accumulates within fertilized sea urchin eggs, irrespective of large changes in concentration in the external medium. This resembles the constancy in the rate of oxygen consumption of cells, over a wide range of different oxygen tensions, as long as the tension exceeds a certain minimal value. Apparently the primary factor which determines the rate of phosphate entry into fertilized eggs is the rate at which phosphate is bound or combined within the cells.

The question arises as to the importance of phosphate in sea water for the development of the eggs. Both Loeb (1907) and Herbst (1898) reached the conclusion that phosphate in the medium is not necessary for normal development.

However, these investigators used artificial sea water prepared from the individual salts, which had not been specially purified, and the only criterion for the absence of phosphate was the lack of a positive test with a molybdate method which was far too insensitive. Herbst used highly dilute sea urchin egg suspensions in the order of several drops of eggs to a beaker of sea water. In view of the data presented in this paper, under such conditions even a trace of phosphate would have been sufficient to adequately supply the eggs. Eggs with a very low intracellular inorganic phosphate content, such as the eggs of *S. purpuratus* (Chambers and White, 1949) and *S. dröbachiensis* (Chambers and Mende, 1953a, 1953b), may have more need for an external source of phosphate than eggs with a high content of inorganic phosphate, such as *Arbacia punctulata* (Chambers and Mende, 1953b), and *Paracentrotus lividus* (Zielinski, 1939).

On the basis of experiments in which the rate of P^{32} uptake by *S. purpuratus* spermatozoa was measured in a suspension containing 1.0 ml. solid sperm/liter sea water, it was found that one ml. of solid sperm takes up P^{32} more slowly than a corresponding volume of unfertilized eggs (Chambers and White, unpublished data). Accordingly, the amount of P^{32} which would enter the few excess spermatozoa attached to the outer surface of fertilized eggs is infinitesimal, compared to the amount actually found to enter the eggs.

Relation to oxygen consumption. The slow rate of P^{32} uptake by unfertilized sea urchin eggs (two to six hours after removal from the ovaries) is observed during the period when the eggs would show a fairly constant and low rate of oxygen consumption (Borei, 1948, 1949). The prominent increase in the rate of uptake of P^{32}, which occurs after sea urchin eggs are inseminated and represents the accumulation of phosphate within the fertilized eggs, takes place during a period when the rate of oxygen consumption increases markedly (Borei, 1948; Tyler and Humason, 1937).

Laser and Rothschild (1939) describe a marked increase in the rate of oxygen consumption of *Psammechinus miliaris* (sea urchin) eggs at 20° C. within the first five minutes after insemination, followed by a fall to the original unfertilized level within ten minutes. During the corresponding period in the eggs of sea urchins, no increase in the rate of penetration of orthophosphate was observed. However, within the first 6 to 7 minutes after insemination at 16° C., a prominent decrease in the concentration of intracellular inorganic phosphate has been noted in the eggs of *S. purpuratus* (Chambers and White, 1949) and *S. dröbachiensis* (Chambers and Mende, 1953b).

The rate of oxygen consumption of sea urchin eggs reaches a maximum some time before first cleavage (Runnström, 1933) and remains fairly constant during the next several cleavages. The rate of P^{32} uptake (at 15° C., for both species of *Strongylocentrotus* eggs) reaches a maximum between 30 to 40 minutes before first cleavage and remains constant thereafter through the first two or three cleavage cycles.

Zeuthen (1949, 1950a, 1950b, 1951) has demonstrated that superimposed on the basic oxygen consumption curves of sea urchin eggs are definite waves of relatively small magnitude, the minima corresponding to the periods of cytoplasmic division, the maxima to the prophases. Although no alterations could be detected

in the rate of P^{32} uptake during the first few cleavages, it should be emphasized that the accuracy of the P^{32} uptake method described in this paper is such that waves of considerably greater magnitude than those described by Zeuthen would not have been detected. However, using a method of much greater accuracy, cyclic variations in the rate of P^{32} uptake have been observed during the later segmentation stages of sand dollar eggs (Chambers, White and Zeuthen in Zeuthen, 1951).

In the experiment carried out using the eggs of *Urechis caupo,* obtained from freshly collected animals, the rate of P^{32} uptake did not increase following fertilization until after the second cleavage. This may be related to the fact that an increase in the rate of oxygen consumption does not occur in these eggs, obtained from freshly collected animals, until during the later cleavage stages (Tyler and Humason, 1937; Horowitz, 1940).

Changes in the rate of oxygen consumption appear to parallel changes in the rate of accumulation of phosphate in the marine eggs studied, at least before and after fertilization and during the early cleavage stages. Following fertilization, the sea urchin eggs accumulate phosphate, since energy from oxidative processes is utilized in the synthesis of organic phosphorous-containing compounds (Chambers and Mende, 1953b).

Summary

1. A method is described for measuring the concentration of radioactive isotope in cells without washing the cells free of the surrounding radioactive medium.

2. P^{32} as orthophosphate penetrates the unfertilized eggs of all species at a slow and constant rate.

3. During the first 10 to 15 minutes following the insemination of *Strongylocentrotus purpuratus* and *S. franciscanus* eggs, the rate of P^{32} uptake is essentially identical to that of the unfertilized eggs. The rate of uptake increases by 15 to 20 minutes, and reaches a maximum by 50 to 90 minutes after insemination. Thereafter, through the first three cleavages the rate remains constant, within the limits of error of the method, as long as the concentration of P in the medium is in excess of 20 μg P/liter.

4. Following insemination of *Urechis caupo* eggs, the rate of P^{32} uptake does not increase. After the second cleavage, however, the rate of P^{32} uptake increases, and a maximum rate has not been attained even after the fourth cleavage.

5. When fertilized eggs containing P^{32} are suspended in non-radioactive sea water, they slowly lose P^{32} to the external medium. On the other hand, no P^{32} is lost from fertilized eggs containing radioactive phosphate when they are washed in non-radioactive sea water.

6. P^{32} is not absorbed to the extraneous coats of fertilized eggs.

7. The rate of penetration of phosphate into the unfertilized and fertilized eggs has been calculated in terms of the μg P entering one ml. eggs/minute. The rate at which phosphate enters fertilized *Strongylocentrotus* eggs is relatively independent of the external concentration, as long as this exceeds 20 μg P/liter. Phosphate enters fertilized *Strongylocentrotus* eggs 86 to 132 times faster that it penetrates the unfertilized eggs.

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